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Protocol

RB PROTOCOL TO ASSESS REDUCTION IN BACTERIAL CONTAMINATION IN INDOOR AIR

Test Microorganism(s):

Staphylococcus aureus (ATCC 6538)
Klebsiella pneumoniae (ATCC 4352), or
Pseudomonas aeruginosa (ATCC 15442)
(*Additional bacteria may be listed.)*

Performing Laboratory:

CREM Co Labs
3403 American Drive
Mississauga, Ontario, Canada L4V 1T4

Sponsor

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Test Guideline

EPA OCSPP 810.2500

Protocol Identification No.
To be identified

May 18, 2021

RB PROTOCOL TO ASSESS REDUCTION IN BACTERIAL CONTAMINATION IN INDOOR AIR

1.0 Objective

1.1 This document describes assessment of a test substance for a temporary reduction in the number of viable bacteria in indoor air to support air sanitization labeling claims.

2.0 Good Laboratory Practice

This study will be conducted in accordance with EPA Good Laboratory Practice (GLP) regulations (40 CFR Part 160). If necessary, an external Quality Assurance Unit may be provided for this study. The following exceptions to EPA Good Laboratory Practice may be noted in the final study report:

For the studies not performed by or under the direction of the Test Facility, including test substance characterization (40 CFR Part 160.105), a Certificate of Analysis for characterization will be appended to the study report.

The computer equipment used in the BSL-2 to capture the room temperature and relative humidity (RH) readings may not be handled in accordance with 40 CFR Part 160. The captured data will be printed following each experiment, initialed and dated, and archived in the study file for permanent storage.

The photography equipment and retention of the electronic form of photos taken during study conduct may not be handled in accordance with 40 CFR Part 160. The photos will be printed, initialed and dated, and archived in the study file for permanent storage.

An external Quality Assurance Unit (QAU) may be engaged to review and certify the quality of the data recorded for the study.

3.0 A glossary and list of abbreviations may be found in Appendix 4.

4.0 Purpose

4.1 The purpose of this study is to evaluate the ability of a test substance to provide a temporary reduction in the number of bacteria in an aerosol chamber to support air sanitization labeling claims.

5.0 Justification for Selection of the Test System

5.1 The study design and test system comply with the U.S. Environmental Protection Agency (EPA) OCSPP 810.2500 Air Sanitizers – Efficacy Data Recommendations (December 2012) with the following exceptions:

3.1.1 An allowance has been inserted for selection of additional non-tuberculous, non-spore-forming bacteria to support additional claims (e.g., *Acinetobacter baumannii*) as desired.

6.0 Scope

6.1 This document outlines the procedure to assess the ability of chemical agents to inactivate representative vegetative bacterial pathogens in indoor air.
6.2 Strict adherence to the protocol is necessary for the validity of the test results. Any deviation from the procedures described here must be documented and justified.

7.0 Experimental Dates

7.1 The proposed experimental start date is {*insert date*}.
7.2 The proposed experimental termination date is {*insert date*}.

8.0 Test Substance Characterization

8.1 In accordance with 40 CFR Part 160.105, test substance characterization as to identity, strength, purity, solubility, and composition, as applicable, will be documented in this study. The stability, if appropriate, will be determined prior to or concurrently with this study.
8.2 The sponsor will report if the characterization and stability studies have been performed under GLP by filling up the Test Substance Characterization GLP Compliance Assessment form. Which will be appended to the study report. Characterization and stability studies which are not performed under GLP will be exempted in the GLP compliance statement.
8.3 A Certificate of Analysis summarizing the chemical characterization will be appended to the study report. Characterization and stability studies not available at the performing laboratory will be exempted in GLP compliance statement.
8.4 The test substance will be prepared in accordance with EPA Lower Certified Limit Guidance in 810.2000 (2018).
8.5 See Section 23.0 for chemical analysis conducted during testing.

9.0 Test Substance

9.1 Test Substance Name: {Insert name}
9.2 Test Substance Batch Number: {*For testing of the required species of bacteria, three batches will be identified in this section. For testing of additional types of bacteria, two batches will be used*.}
9.3 Test Substance Storage Conditions: {Insert conditions} Test Substance Preparation: {*Insert preparation instructions including required dilutions and diluents*}
9.4 For concentrates, the diluent used in testing and described in labeling will be consistent with EPA guidance in 810.2000 (February 2018).
9.5 Test Substance Application Method: {Insert air treatment instructions. The labeled use instructions will be based upon the technique used during testing (e.g., for an aerosol, press the trigger of the can spray continuously for “x” seconds towards the center of the room aiming at the ceiling to treat the air.)}
9.6 Test Substance Exposure Time(s): {*Insert exposure time(s)* (e.g., 5, 10, 60 mins)}: The exposure or dwell time of the released chemical in the air will be measured and documented.
9.7 The air temperature (e.g., 20-25°C) and relatively humidity (RH; e.g., 50±5%) in the chamber during each experiment will be measured and recorded using a datalogger.

9.8 Test Substance Retention is the responsibility of the Sponsor. All unused test substance will be discarded following study completion unless otherwise indicated by the Sponsor.

9.9 See Section 23.0 for chemical analysis to be conducted during the testing.

10.0 Labware:

10.1 All items are to be sterile and disposable; they are available from any supplier of scientific labware.

10.2 Micropipettes with appropriate tips to accurately deliver 20, 100 or 1000 µL volumes

10.3 Screw cap tubes – 2 mL

10.4 Screw cap tubes - 50 mL

10.5 Screw cap tubes -15 mL

10.6 Petri plates -150 X 25 mm

10.7 Petri plates -100 X 15 mm

10.8 Serological Pipettes – 1, 5, 10 and 25 mL capacity

11.0 Personal protective equipment (PPE; all items listed below are available from suppliers of laboratory safety gear).

11.1 Safety glasses

11.2 Laboratory gloves

12.0 General Solutions and Reagents

13.1 Deionized distilled water (DDW) or equivalent, for making reagent solutions and media.

13.2 Trypticase soy broth (TSB), to culture the test bacteria.

13.3 Letheen Broth (LB) agar or TSA with proper neutralizer to recover the bacteria from control and test samples and for conducting sterility tests.

13.4 Antifoam A concentrate (Sigma-Aldridge, St. Louis, MO; Cat. # A-5633), is added to the bacterial suspension to be nebulized to reduce frothing. This autoclave-sterilizable silicon-based item is commonly used in fermentation systems and is devoid of bactericidal or bacteriostatic activity.

13.5 Dulbecco's PBS.

13.0 Soil load (ASTM International 2013; OECD 2013; Springthorpe and Sattar, 2007).

13.1 The soil load for incorporation into the bacterial suspension to be nebulized consists of a mixture of the following thawed stock solutions in phosphate buffered saline (PBS; pH 7.2±0.2):

13.1.1 0.5 g of yeast extract in 10 mL of PBS.

13.1.2 0.5 g of bovine serum albumin (BSA) in 10 mL of PBS.

13.1.3 0.04 g of bovine mucin in 10 mL of PBS.

13.2 The stock solutions of all three components of the soil load are sterilized by passage through a syringe-mounted (25 mm diameter) polyethersulfone (PES) membrane (0.22 μm pore diam.).

13.3 All three solutions are then aliquoted as 1.5 mL volumes and stored at -20±2°C with a shelf-life of at least one year. For short-term storage, the vials can be kept at 4±2°C for no longer than 90±5 day

14.0 General equipment

14.1 Air displacement pipettes (Eppendorf or equivalent) with tips to dispense 100 to 1000 µL volumes.

14.2 Analytical balance, to weigh chemicals and to record and standardize inoculum delivery volumes as well as calibration of pipettes.

14.3 Centrifuge to attain speeds of 3,000xg or higher, to allow for the sedimentation of the test bacteria for concentration or washing, or both.

14.4 Freezer: At -20±2°C is required for the storage of media, reagents and additives.

14.5 Deep freezer: At -70°C or lower to store the stocks of test microorganisms.

14.6 Incubator: To maintain a temperature of 36±1°C for the culture of the test microorganisms and also for sterility testing.

14.7 Biological safety cabinet (BSC), Class II (Type A): Certified; please refer to *Biosafety in Microbiological and Biomedical Laboratories* (CDC 2020) for proper maintenance and operation of this piece of equipment. The performing laboratory must have its own standard operating procedure (SOP) for the certification, maintenance, and operation of such devices.

14.8 Refrigerator: at 4±2°C for storage of media, culture plates and reagents.

14.9 Autoclave, to sterilize culture media, reagents and waste.

14.10 Vortex mixer, to homogenize bacterial suspensions.

14.11 Balance to weigh the nebulizer before and after spraying of the microbial suspension.

15.0 Examples of Specialized equipment (Further details are given in Appendix 1)

15.1 A slit-to-agar (STA) programmable sampler: For event-related collection of bacterial aerosols (e.g., Pinpoint Scientific Ltd, 1st Floor, North Road, Bridgend Industrial Estate, Bridgend, , CF31 3TP; (sales@pinpointscientific.com).

15.2 Six-jet Collison nebulizer: To generate microbial aerosols in the respirable range of 0.5-5.0 µm (e.g., CH Technologies., 778 Carver Ave, Westwood, NJ 07675, www.chtechusa.com); cylinder of extra-dry compressed air with pressure regulator and a back flow preventer.

15.3 Volatile-gas detector with a gas leak probe: To check for any air leaks from the chamber (e.g., Model BT-45; Quantum Instruments, Garden City, NY).

15.4 Air temperature and RH meter: To monitor and record the air temperature and RH in the aerosol chamber via a wireless data logger (e.g., CAS Data Loggers, 8437 Mayfield Rd., Unit 104 Chesterland , OH 44026) ; www.dataloggerinc.com/).

15.5 Magnehelic: To detect any pressure differential between the inside and outside of the chamber (Figure 1) (e.g. ITM instrument Inc. 16975 Leslie St. Newmarket, ON L3Y 9A1).

15.6 A muffin fan to evenly distribute the aerosols inside the chamber and to keep them airborne during testing.

15.7 Device for the collection of the test substance from the chamber air

16.0 Bacterial species for testing.

16.1 Since contamination of stock cultures can negatively impact the test data, it is crucial to abide by the highest standards of GLP during all manipulations and handling of stock and working cultures.

16.2 All manipulations of the test microorganisms must be performed in accordance with the biosafety practices stipulated in the relevant SOPs of the performing laboratory.

16.3 In its guidance document (EPA 810.2500 2012), EPA recommends the use of the two bacterial species given in Table 1. Testing against *Pseudomonas aeruginosa* will replace *Klebsiella* when use in healthcare settings is desired per 810.2200 (2018).

16.4 Appendix 2 lists the growth characteristics/morphology of the bacteria to be used.

Table 1. Test Bacteria

Microorganisms
Staphylococcus aureus (ATCC 6538)
Klebsiella pneumoniae (ATCC 4352) *or* *Pseudomonas aeruginosa* (ATCC 15442)
Additional Bacteria (e.g.*, Acinetobacter baumannii*)

All of these can be grown in TSB and recovered on LB agar or TSA with the proper neutralizer.

17.0 Maintenance, passage and storage of test bacteria:

17.1 Obtain standard strains of the bacteria (lyophilized) to be used in the testing from a reputable source such as the American Type Culture Collection (ATCC). Every 18 months (or sooner if the quality of the stock culture is compromised) prepare new stock cultures of the test bacteria from the lyophilized material. In case such a stock is unavailable, order a fresh culture directly from a source such as the ATCC.

17.2 Use the following procedures to initiate and maintain in-house stocks of the cultures.

17.3 Culture Initiation

17.3.1 Wipe the outside of the ampule/vial of the freeze-dried culture with a towelette prewetted with 70% (v/v) ethanol and open it inside a laminar flow hood.

17.3.2 Resuspend the freeze-dried material in 1.0 mL of sterile TSB.

17.3.3 Using a pipettor with a sterile pipette tip place 0.1 mL of the rehydrated suspension into each one of two 10.0 mL tubes containing 5.0 mL of sterile TSB. Mix well by shaking.

17.3.4 Streak a loopful of the suspension onto two 100 mm diameter TSA plates (predried to remove any accumulated water on the surface of the agar) to obtain isolated colonies, and incubate the plates at 36±1ºC for 18±2 h.

17.3.5 Observe the plates for growth and typical colony morphology of the bacterium. For example, the colonies of *S. aureus* should appear round, convex, entire, glossy, golden-colored colonies ~2 mm in diameter; the colonies of *K. pneumoniae* should look round, entire, convex, and creamy-white with a diameter of 3-4 mm; the colonies of *P. aeruginosa are* mucoid, circular and raised with an undulate margin and a blue-green pigment surrounding the colony with a colony diameter of 3-4 mm.

17.3.6 Prepare a smear from an isolated colony, Gram-stain it and observe the smear microscopically under an oil-immersion objective (1000X) to ascertain that the Gram-reaction/morphology of the bacterial cells is correct. For example, the cells of *S. aureus* should appear as cocci in singles, pairs, tetrad and grape-like clusters with a deep purple color (Gram-positive), those of *K. pneumoniae* and *P.*

aeruginosa should be randomly-distributed and typically occur as straight rods with rounded or slightly pointed ends, found singly, in pairs or in short chains with pinkish-red color (Gram-negative).

17.3.7 If required, subject the culture to additional characterization by biochemical and/or molecular means (Appendix 2).

17.4 Cryopreservation of cultures: Prepare a broth culture of the desired bacterial species by inoculating with a flamed loop a colony from the TSA plate into 9.0 mL of TSB and incubate the tube at 36±1°C for 18±2 h. Add to this broth culture 1.0 mL of autoclave-sterilized glycerol, shake well and reincubate for 2 h before mixing well and aliquoting into labelled (with indelible ink) cryovials each displaying the source, scientific name, passage number, lot number and date of storage of the test bacterium. Store the vials at -70ºC or below for no longer than 18 months.

17.5 Test Culture Preparation:

17.5.1 Thaw frozen test culture quickly by holding the vial under running warm water from a tap or by immersing it in a waterbath at 45±1°C.

17.5.2 Inoculate 10 mL of TSB with 100 µL the thawed stock culture and incubate at 36±1ºC for 18±2 h. **This is Passage #1**

17.5.3 To prepare a "Refrigerated Stock Culture", inoculate 10 mL of TSB with 100 µL of the culture and incubate at 36±1ºC for 18±2 h. Place at 4±2°C for no longer than 7 days. **This is Passage #2**

17.5.4 For preparation of working culture, Inoculate 100 µL of the "Refrigerated Stock Culture" into 10 mL of TSB and incubate at 36±1ºC for 18±2 h.

17.5.5 Using a Vortex-style device, resuspend the culture for 3-4 s. When working with *P. aeruginosa* cultures, remove any pellicle prior to resuspension.[1].

17.5.6 The culture should be adjusted to deliver approximately 1.6×10^4 cfu/m^3- 1.0×10^5 cfu/m^3 such that recovery from the baseline sample of parallel untreated control is equivalent to 4.2 – 5.0 $\log_{10}$ CFU/m^3.

17.5.7 To prepare the fluid for each nebulization into the aerobiology chamber, add to the nebulizer reservoir 0.75 mL BSA, 1.05 mL yeast extract, 3.0 mL mucin, 50 µL of the test bacterial suspension and 10 µL of Antifoam A to 10.14 mL of Dulbecco's PBS.

17.5.8 Assay the fluid to be nebulized for CFU before and after nebulization by making five 10-fold dilutions (e.g. add 100 µL to 900 µL of PBS). Plate appropriate dilutions
in duplicate by pour/spread plating or filtration. If using the Miles & Misra (1938) plating technique, place five 20-µL droplets from each the last three dilutions on a 100 mm plate of TSA with a predried agar surface.

17.5.9 Incubate plates for 18±2 h at 36±1ºC. Record the CFU and also observe them for any extraneous microbial contamination. The test data would be invalid in case any contamination is detected.

18.0 Basic design of the aerobiology chamber:

[1] Please refer to SOP No. MB-05-13 (12.2a) from BEAD Lab of the U.S. EPA (http://www.epa.gov/pesticides/methods/atmpmethods/MB-05-08.pdf).

18.1 Appendix 1 summarizes the details on the specialized pieces of equipment used in the protocol. The equipment and materials listed are examples only and may be substituted with equivalent items from other sources.

18.2 The aerosol chamber (Figure 1) is an enclosure with a volume of 900.0 ft^3 (25.00 M^3) located inside a clean room with negative pressure and controlled access. The chamber's walls are made out ot wipe-able, solid coroplastic sheeting (https://www.homedepot.com/p/Coroplast-48-in-x-96-in-x-0-157-in-White-Corrugated-Plastic-Sheet-CP4896S/205351385) affixed to a framed structure to represent the walls to maintain an airtight seal. Sealable ports, window and door provide access to the inside of the chamber for maintenance and to place and remove any monitoring devices to be used. The walls should be grounded properly to dissipate any static electricity that may accumulate.

18.3 While the chamber can be used with all major classes of microorganisms at biosafety levels (BSL) 1 and 2, the CDC guidelines (CDC 2020) recommend that extra safety precautions and operational requirements be in place for work with experimental aerosols of all such microorganisms. The negative pressure and controlled access to the chamber are designed to provide this additional protection, turning is into 'BSL-2+' facility.

18.4 In accordance with the current EPA guidelines (2012), the chamber does not permit any air exchanges; nor does it contain any furniture or fixtures in accordance with EPA 810.2500 study design description. Furniture and fixtures were not placed in the chamber inside of the BSL facility due to biosafety and decontamination concerns over the multiple test dates over a long period.

18.5 The chamber's internal environment is monitored throughout an experiment with a wireless relative humidity (RH)/air temperature sensor/data logger system (e.g., CAS Data Loggers, 8437 Mayfield Rd., Unit 104 Chesterland , OH 44026); www.dataloggerinc.com/) and recorded on cloud for subsequent download and analysis.

18.6 To assess the airborne survival of the test bacteria or to determine the activity of any air sanitization technology, the air in the chamber is sampled at the rate of 1 ft^3 (28.3 L)/minute using an externally-placed slit-to-agar air (STA) sampler with a built-in vacuum pump This programmable device can be set to operate for a minimum air sampling time of 30 seconds to as long as five hours depending on the STA model, and the actual length of sample collection time will be determined by the anticipated load of viable bacteria in the air of the chamber. The air exiting the sampler is discharged directly into a HEPA incorporated in the device or into the BSL-2 facility's HEPA-filtered exhaust system. For the baseline value, the concentration of the test bacteria in the nebulizer fluid should be adjusted to achieve a minimum of 4.2 $\log_{10}/m^3$ to a maximum of 5.0 $\log_{10}$ CFU/m^3 at the start of the treatment. An STA has been chosen due to its higher efficiency for sampling airborne microorganisms (Borges et al., 2021).

18.7 Between experiments, the air inside the chamber is replaced with fresh air using a vacuum pump and the exiting air directly discharged into a BSC located in the clean room for a minimum of one hour.

18.8 Start and stop times (clock times) will be recorded for the application of the treatment to the air. The official exposure period or contact time begins upon completion of the release of the test substance which should begin after the nebulizer has completed the 10-minute release of the test bacteria, five minutes for stabilization of the aerosols and the 2 minute pre-treatment air sample is taken.

18.9 Any spray device can also be placed inside the chamber and activated from the outside or by accessing it with the gloves affixed to the chamber (Figure 1).

18.10 A magnehelic is affixed to the outside of the chamber to visually indicate on a continuing basis pressure differential between its internal and external atmospheres. Any pressure differential would be regarded as indicative of a breach in the integrity of the chamber resulting in the immediate termination of the test.

18.11 The exposure period (contact time) may vary with the Test Substance. The same exposure period will be used to evaluate each lot of a Test Substance and controls. The air will be sampled for the same duration and at the same intervals for each lot of a Test Substance but no fewer than three air samplings per lot per microorganism will be collected.

18.12 The air may be sampled for different durations and after different intervals for Test Substance and Controls to recover countable CFU on sampling plates and reduce the detection limit as much as possible. No fewer than three air samplings per lot per microorganism per chamber run will be collected for both Test substance and Controls. Each test lot will be evaluated in three runs of the chamber for each of the two bacterial species.

19.0 Experimental Design: A generic sequence of the main steps in the operation of the chamber is given in the Flowchart below.

Flowchart.

Switch on circulation fan;

Check environmental parameters and adjust as needed

↓

Run an air sampler for 2 minutes for background contamination;

Nebulize bacteria for 10 minutes and allow to stabilize for 5 minutes

↓

Collect another 2-minute Baseline air sample to confirm 4.2 -5.0 log CFU/m^3

↓

Introduce test substance inside the chamber – this step is omitted for the parallel untreated control and bacterial stability in air experiments

↓

Collect air samples for bacterial/chemical analyses at intervals to support claims

↓

Flush chamber with fresh air for at least one hour to decontaminate it;

Repeat for additional lots/controls

20.0 Method for Control of Bias: None

21.0 Operation of the aerobiology chamber

21.1 Actuate the 'muffin' fan (e.g. Cooltron AC Fan, Model FA8038B11T7-51, 80x80x38mm,7Blds,115VAC,50/60Hz,11/9W, 26/31CFM,) placed on the floor of the chamber directly underneath the nebulizer inlet pipe (Figure 1) 10±2 minutes prior to nebulization of the bacterial suspension. Leave the fan on for the duration of a given test to maintain uniform distribution of the aerosolized particles in the air inside. Between experiments, wipe the outside of the fan with 70% (v/v) ethanol for decontamination.

21.2 Check temperature and RH. Adjust if needed.

21.3 Connect the inlet of the STA sampler to a PVC pipe (ID 2.0" or 5.0 cm) which extends into the center of the chamber (Figure 1).

21.4 Place a 150 mm diam. disposable Petri plate with LB agar or equivalent growth medium inside the sampler. All agar plates used during testing will be equilibrated to room temperature and the surface of the medium dried prior to use. Collect a 2-minute sample using a STA air sampler to measure the background bacterial contamination prior to test initiation. After collection, retrieve and incubate the plate alongside the test plates.

21.5 Attach an externally placed six-jet Collison nebulizer (Appendix 1) to the port on the chamber, connect the nebulizer to a compressed air cylinder (Figure 1) and then adjust the air pressure to 25 pounds/square inch (PSI) to nebulize the bacterial suspension for 10 minutes. Allow the bacteria to circulate and stabilize for 5 minutes.

21.6.1 The level of bacteria in the fluid to be nebulized and the volume nebulized should be previously determined to obtain recovery plates with countable numbers. The sampling times and nebulized fluid should be predetermined to achieve a minimum of 4.2 $\log_{10}$ to a maximum of 5.0 $\log_{10}$ CFU per m^3 in the baseline.

21.6.2 Any plates with colonies which are too-numerous-to-count (TNTC) will invalidate the corresponding sampling point. For the plate of the first sampling point with no visible growth, use a value of 1.0 CFU to take into account the maximum error in the detection limit of STA air sampler.

21.6.3 Weigh the nebulizer before and after nebulization to determine the volume of fluid nebulized. Each gram of weight is regarded as equal to 1.0 mL of the fluid.

21.6.4 At the end of each experiment, retrieve, decontaminate, clean and autoclave sterilize the pipe and the quick connect attached to the nebulizer.

21.7 Place another 150 mm diameter disposable Petri plate with LB agar or equivalent growth medium (agar surface predried) inside the sampler. Collect an air sample to measure the baseline bacterial level in the chamber ("Baseline"). This value serves as

the parallel, untreated control. After collection, retrieve and incubate the plate alongside the test plates.

21.8 After Baseline sampling, the Test Substance is sprayed into room air from a spray can to treat the air. The can will be activated for the desired length of time by pressing its button via the built-in access gloves (Figure 1). Shake test aerosol spray-can well before use. Hold can upright, press button and spray towards the center of the aerobiology chamber in a sweeping motion. The start and stop times (clock times) will be recorded for the application of the treatment to the air. The official exposure period or contact time begins upon completion of the release of the test substance.

21.8.1 Air treatment is omitted when conducting the Bacterial-Stability-in-Air Control. This control is conducted to measure the survival and settle rate of the test microorganisms in the chamber over the test period.

21.9 After completing the release of the test substance start sampling the air continuously following Table 2. After incubation, each plate should be divided into 4 sections and the CFU in each section will be counted and recorded.

Table 2: Sampling Duration Example for the Test Substance

Samples	Sampling Duration (minutes)	Four Sections on each recovery plates (minutes)			
		#1	#2	#3	#4
Baseline**	-2 to 0	Section (Sampling time point) minutes			
1	0*-10	0-2.5 (1.25)	2.5-5 (3.75)	5-7.5 (6.25)	7.5-10 (8.75)
2	10-20	10-12.5 (11.25)	12.5-15 (13.75)	15-17.5 (16.25)	17.5-20 (18.75)
3	20-30	20-22.5 (21.25)	22.5-25 (23.75)	25-27.5 (26.25)	27.5-20 (28.75)
4	30-45	30-33.75 (31.875)	33.75-37.5 (35.625)	37.5-41.25 (39.375)	41.25-45 (43.125)
5	45-60	45-48.75 (46.875)	48.75-52.5 (50.625)	52.5-56.25 (54.375)	56.25-60 (58.125)

***** Time 0 start after finishing the application of the test substance

** Sampling for Baseline can be shorter to get a countable CFU on plates if the STA machine has the capability.

20.9.1 When conducting the Bacterial-Stability-in-Air Control use Table 3 to for sampling time and duration

Table 3: Sampling Duration for the Test Substance

Samples	Sampling time point (minutes)	Sampling Duration (minutes)
Baseline	0	-2 to 0
1	10	9-11
2	20	19-21
3	30	29-31
4	45	44-46

5	60	59-61

21.10 In order to calculate the time in which the test substance demonstrate 3 $\log_{10}$ reduction, CFU/m^3 will be calculated for each sampling time point using the counted CFU on the corresponding section following the formula presented in Appendix 2. The $\log_{10}$ CFU/m^3 will be plotted for each sampling time point for control and efficacy tests. The time in which the test substance demonstrates 3 $\log_{10}$ reduction in CFU/m^3 will be calculated following procedure explained in Appendix 2.

21.11 Using a towelette soaked in 70% (v/v) ethanol, wipe the outside as well as readily accessible inside surfaces of the sampler after each test. Similarly, decontaminate the outside and inside surfaces of the air sample collection pipe (Figure 1).

22.0 Chamber Testing Schedule

22.1 Appendix 5 illustrates an example testing schedule for 2 lots of test substance on the 3 required test bacterial strains with all required controls.

23.0 Confirmation of neutralization of active ingredient(s) prior to testing

23.1 Depending on the type and concentration of the active ingredient(s) under test, a neutralizer (with one or more components) is incorporated into the agar medium for air sampling to immediately arrest bactericidal and/or bacteriostatic activity.

23.2 To validate the activity of such a neutralizer, introduce the test substance lot with the highest level of the active(s) into the chamber.

23.3 Collect a 30-minute air sample using an STA sampler containing a plate with the recovery agar medium.

23.4 Within 30 minutes after collection of the air sample, spread over the agar plate 100 µL of the test bacterial suspension diluted to contain $\leq$100 CFU per plate.

23.5 For controls, (a) use the same procedure to inoculate two unexposed plates containing the neutralizer, and (b) one agar plate from the same lot as sterility control. Incubate plates at an appropriate temperature and observe them after 18±2 hours of incubation and continue incubation for an additional three days. Count and record the CFU. The performance criteria are no more than a 20% difference in the numbers of CFU on (a); and (b) should be free from any visible growth.

23.6 The neutralizer is regarded as effective if the number of CFU on the test plates is within 80-120% of the CFU count on the control plate (OECD 2003). Any bacterial or fungal growth on the sterility control plate would invalidate the test.

24.0 Collection and analysis of test chemical(s) in the air of the chamber

24.1 Collect and analyze air samples from a run of the chamber without the test strain for each lot of test substance using a validated chemical analysis method at the same time points and durations used for bacteriological samples collected during treatment. For example, the concentration of Dipropylene Glycol – DPG and Triethylene glycol - TEG in air would be analyzed by Gas Chromatography similar to NIOSH Method # 5523 using a validated method. One chemical collection test will be performed per lot.

25.0 Incubation

25.1 Incubate all test and control plates at 36±1ºC and observe them after 18±2 hours.

25.2 Incubate all plates at the end of 18±2 hours of incubation for an additional three days to allow injured or stressed bacterial cells to grow and form visible colonies (Springthorpe and Sattar 2007).
25.3 Enumerate survivors and calculate bacterial survival/reductions.
25.4 See Appendix 3 for calculations.

26.0 Quality Control

26.1 Sterility Control: One plate of growth media will be incubated alongside the test. All reagents will be evaluated by plating 1.0 mL on growth media and incubated alongside the test. The acceptance criterion for this control is lack of visible growth.

26.2 Viability Control: The growth/neutralizing media will be challenged in duplicate with <100 CFU bacteria and incubated alongside the test to confirm the media can support the growth of low numbers.

26.3 Purity Control: A streak plate will be prepared of the test culture and incubated alongside the test to confirm use of a pure culture. The acceptance criterion for this control is the demonstration of a pure culture.

26.4 "Bacterial Stability-in-Air" Control: This control is conducted as described in Section 20.0 omitting treatment with the test substance (Section 20.9). This control measures the survival and settle rate of the test microorganisms in the chamber over the test period. There are no acceptance criteria for this value. This value is used in the calculation of the reduction performance. This study is conducted at least three times for each bacterial species

27.0 Statistical Analysis

27.1 The data will be subjected to appropriate statistical analyses for the preparation of the final project report. Such analyses will include, at a minimum, calculation of standard deviations (SD).

27.2 Method for Control of Bias: None

28.0 Study Acceptance Criteria:

28.1 Test Substance Performance Criteria: After correction for bacterial settling and natural biological decay, the test substance must demonstrate ≥99.9% (3 $\log_{10}$) reduction in the viability of the bacterial species over the parallel untreated control.

28.2 Baseline Acceptance Criteria: The control recovery must demonstrate a minimum of 4.2 $\log_{10}$ to a maximum of 5.0 $\log_{10}$ CFU/m^3 at the start of the treatment for a valid test.

28.3 The maximum allowable contact time to support an air sanitization claim should not be longer than 5 minutes for a >3 log10 reduction in the level of viability of all species of bacteria to be tested.

29.0 Control Acceptance Criteria:

29.1 All sterility controls must be free of any visible growth.
29.2 Viability Control must demonstrate growth in all media with <100 CFU/plate.
29.3 Purity Control must demonstrate a pure culture.
28.4 Neutralization Validation: The mean number of CFU on the plate unexposed to the test substance and those on the plate exposed to the test substance must be within 50%.

29.5 Magnehelic readings must indicate no leaks in the chamber during an experiment.
29.6 Temperature and RH readings must stay within range required for the test.

30.0 Retesting Guidance

30.1 For tests where the product passes and the mean Baseline value is above 5.0 $\log_{10}$ CFU/m^3, no retesting is necessary. For tests where the product fails and the mean *Baseline* is above 5.0 $\log_{10}$ CFU/m^3, retesting may be conducted. For tests where the product fails and the mean baseline is less than 4.2 $\log_{10}$ CFU/m^3, no retest is required

31.0 Protocol Changes

31.1 Protocol changes or revisions, if needed, will be documented including the reason for the change, signed/dated by the Study Director and Sponsor, and described in the study report. SOPs used in the study will be the effective version at the time the study will be conducted. Changes in SOPs not required by the protocol will be documented in the raw data and approved by the Study Director.

32.0 Study Report:

31.1 The study report will include all elements listed in OCSPP 810.2000 (2018), 810.2500, 40 CFR Part 160.185, and EPA Pesticide Registration Notice 2011-3.

33.0 Study Retention:

33.1 All original raw data for this study will be archived at the Test Facility until study completion including all handwritten raw data for control and test substances (e.g. notebooks, data worksheets, and calculations), protocol amendments/deviations. SOP deviations, study specific correspondence, original signed protocol, and the signed study report.

33.2 All facility records for this study will be archived at the Test Facility until study completion including SOP, referenced methods, QA reports, equipment logs, equipment calibration and maintenance logs, reagent preparation and quality control records, personnel training, education, and experience records, etc.

33.3 Following study completion, the Test Facility will keep all raw data and certified copies of facility records for 5 years before transferring them to the RB GLP Archives (Montvale, NJ).

30 Approval Signatures

Name of Sponsor:
Name of Sponsor's authorized representative:
Title:

_____

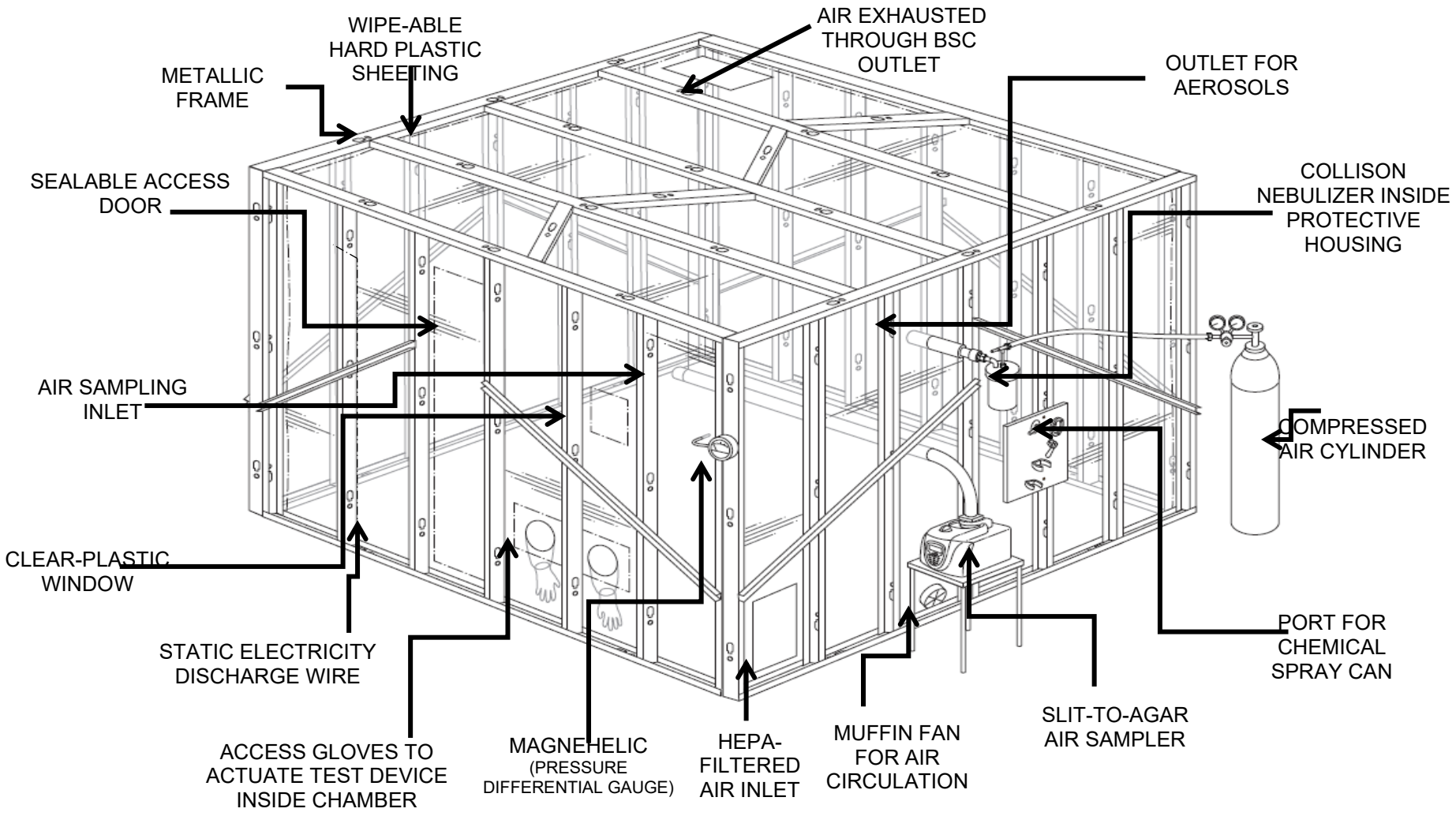
Signature of Sponsor's authorized representative Date

Name of Performing Laboratory:

Name of Study Director:
Title:

Signature of Study Director Date

Figure 1: Aerobiology Test Chamber with Essential Components (900 ft^3 or 25 M^3)



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Appendix 1

Examples of additional specialized pieces of equipment required

Equipment (Cat. #)	Manufacturer	Purpose	Design features/Justification for use	Maintenance/Operation	Illustration
1. Collison six-jet nebulizer (Model: MRE CN24/25)	CH Tech., Westwood, NJ 07675; www.inhalation.org	Generate airborne particles in the respirable range (0.1-5.0 µm in diam.).	Air at a pressure of about 25 psi (172.37 kPa) from a pump or compressed air cylinder is needed for operation. The glass reservoir receives 15 mL of the bacteriophage / virus-soil mixture and it is weighed before and after nebulization to determine the volume aerosolized and may be used to estimate the number of PFU introduced into the chamber air. The liquid to be nebulized contains antifoam to reduce excessive foaming during nebulization. This type of nebulizer is favored in microbial aerobiology due to its versatile and well-characterized nature. The size range of particles generated by it not only are in the respirable range, but the droplet nuclei arising from them can remain suspended in air for periods long enough to study biological decay and/or the impact of physical or chemical agents on the viability of airborne infectious particles.	The entire unit, which is made of metal & glass, can be readily washed and autoclave-sterilized between uses. The nebulizer may be placed inside a shatter-proof plastic housing as extra workplace safety precaution in case of any leakage or breakage of the nebulizer's glass container. This device does not require any periodic recalibration.	
2. RTR-500 series data logger RTR-503L for relative humidity & air temperature (cat. RTR-503L)	CAS Data Loggers, 8437 Mayfield Rd., Unit 104,Chesterland , OH 4 4026 www.dataloggerinc.com	Remotely sense and record relative humidity (RH)/air temperature in the chamber.	RH and air temperature are among the crucial factors affecting microbial survival in air. They may also influence the efficiency of any air decontamination technology being assessed. The recorder is designed to send data wirelessly to a remote computer at an adjustable time interval. A 5-minute interval is used for data capture.	This device requires yearly recalibration by the manufacturer.	

3. Sampler slit-to-agar (STA) sampler.	Pinpoint Scientific Ltd, 1st Floor, North Road, Bridgend Industrial Estate, Bridgend, , CF31 3TP; sales@pinpointscientific.com). Or Particle measuring system 5475 Airport Blvd Boulder, Colorado 80301 USA T: +1 303 443 7100, +1 800 238 1801 W: www.pmeasuring,com	Collect airborne bacteriophage / virus on a timed and event-related basis.	A disposable plastic Petri plate (150 mm diam. X 15 mm in height) with nutrient agar (75 mL) is placed on the sampler's rotating platform to collect aerosols by impingement, and the distance between the bottom surface of the slit and the top surface of the agar is automatically adjusted for optimal particle impingement. The sampler has a built-in vacuum pump to draw in the air to be sampled, and a timer to permit adjustment of air sampling duration from a minimum of 2 minutes to a maximum of 5 hours. At the end of the sampling time, the plate is removed and incubated. The sampler automatically controls the air sample collection rate at 28.3 L/minute. It can also record sampling data for subsequent download to a computer.	Does not require any sterilization between uses but a simple wipe-down with a disinfectant-soaked towelette. Before each use, the air-inlet must be inspected to ensure that the slit is free of any obstructions. The volume of the agar in the Petri plate must be precisely measured to maintain a specific distance between the surface of the agar and the bottom of the slit. The agar surface must also be free of any water drops before placement in the sampler. The sampler has a built-in tubular HEPA filter at the exhaust with a flow-sensor to indicate when the filter needs replacement. This device requires yearly recalibration by the manufacturer.	
4. Volatile gas leak detector and probe	Quantum Instruments; Model BT-45	To detect any leaks in the chamber.	The device could detect either hydrogen gas from a cylinder or glycol introduced into the chamber using a fogger.	Replace battery when stabilization beeps are prolonged past 1 minute.	
5. Muffin fan	Cooltron AC Fan, Model FA8038B11T7-51, 80x80x38mm,7Blds,115 VAC,50/60Hz,11/9W, 26/31CFM, Nidec Alpha V, TA300, Model A31022-20, P/N: 933314 3.0 inch/7.62 cm diam.; output 30 CFM	To keep the aerosolized materials uniformly suspended inside the chamber	Relatively small size with sufficient air flow to keep the bacteriophage/virus and the chemicals suspended in air	Requires wiping of external surfaces with 70% (v/v) ethanol	

6. Equipment and accessories to collect samples of the test chemical from the chamber.	Calibrator: chek-mate Calibrator, 0.50 to 5 L/min, with NIST Certification, catalog # 375-0550N https://www.skcinc.com/products/chek-mate-calibrator-050-to-5-l-min-1 Pump: Universal 44XR Sample Pump Single Kit, catalog # 224-44XRXD https://www.skcinc.com/products/universal-44xr-sample-pump-single-kit-1 Tubes: Sorbent Tubes, XAD-7 OVS, catalog # 226-57 https://www.skcinc.com/products/sorbent-tubes-xad-7-ovs-1	To collect air-sample for analysis of active(s) .	Air-sampling pump with air-collection tube.	Pump flow-rate needs to be calibrated. For maintenance, follow the instructions provided by the manufacturer.	

APPENDIX 3

Baseline Concentration:

Considering that the sampled air has the same bacterial concentration as the air in the chamber, we get the following equation

$$Baseline\ concentration = \frac{CFU\ on\ the\ sampled\ plate}{Volume\ of\ the\ sampled\ air}$$

(1)

As the volume of the air sampled relates to the sampling rate and the duration of sampling, Equation (1) gives us the baseline concentration as follows:

$$Baseline\ concentration = \frac{CFU\ on\ the\ sampled\ plate}{\text{sampling rate} \times duration\ of\ sampling\ in\ minutes} \quad (2)$$

The STA sampler samples air at the rate of 0.0283m^3/min. For example, if there are 2516 CFU on the baseline plate with a 2-minutes sample, the baseline concentration can be calculated as follows:

$$Baseline\ concentration = \frac{2516\ CFU}{0.0283\ \frac{m^3}{min} \times 2\ min} = 44452.30\ CFU/m^3$$

which is equal to 4.65 $\log_{10}$ CFU/m^3

Bacterial concentration in chamber air:

For the *n*th sampling plate, similar to equation (2) we can write:

$$Bacterial\ concentration\ in\ the\ chamber\ air = \frac{CFU\ on\ the\ sampled\ plate}{\text{sampling rate} \times duration\ of\ sampling\ in\ minutes} \quad (3)$$

Removal of the air from the chamber due to collection of each sample dilutes the bacterial concentration. The correction factor required to address this is defined as:

$$Dilution\ Correction\ Factor\ for\ nth\ sampled\ plate = \frac{Volume\ of\ the\ chamber}{Volume\ of\ the\ chamber - \text{sampling rate} \times duration\ of\ sampling\ in\ minutes * n} \quad (4)$$

Therefore, the total bacterial concentration corresponding to the *n*th sampled plate can be calculated as follows:

$$Corrected\ Bacterial\ concentration\ in\ the\ chamber\ air = \frac{Volume\ of\ the\ chamber}{Volume\ of\ the\ chamber - \text{sampling rate} \times duration\ of\ sampling\ in\ minutes * n} \times \frac{CFU\ on\ the\ sampled\ plate}{\text{sampling rate} \times duration\ of\ sampling\ in\ minutes}$$

For example, if the third 2-minute sampling plate contains 1 CFU, we can calculate the corrected bacterial concentration as follows:

$$Corrected\ Bacteria\ concentration\ in\ the\ room = \frac{24.34\ m^3}{24.34\ m^3 - 0.0283\ \frac{m^3}{min} \times 2\ min \times 3} \times \frac{1\ CFU}{0.0283\ \frac{m^3}{min} \times 2\ min} = 17.79\ \frac{CFU}{m^3}$$

which is equal to 1.2 $\log_{10}$ CFU/m^3

Calculating biological Decay and the Efficacy of a substance

To evaluate the efficacy of a test substance, the rate of biological decay of the challenge bacterial species in the air of the chamber is determined first. This is equal to an untreated parallel control. Then, another experiment is conducted (efficacy test) where the test substance is released into the chamber. Since the initial titers of the two experiments may differ in practice, the data for the untreated parallel control is transformed so that its initial titer becomes equal to the initial titer of the efficacy test.

Figure 2 shows CFU recovery data from the untreated parallel control experiment, transformed untreated parallel control and efficacy experiment. Log_{10} reduction at each sampling time is equal to the vertical distance between the transformed untreated parallel control line and the line for the efficacy test.

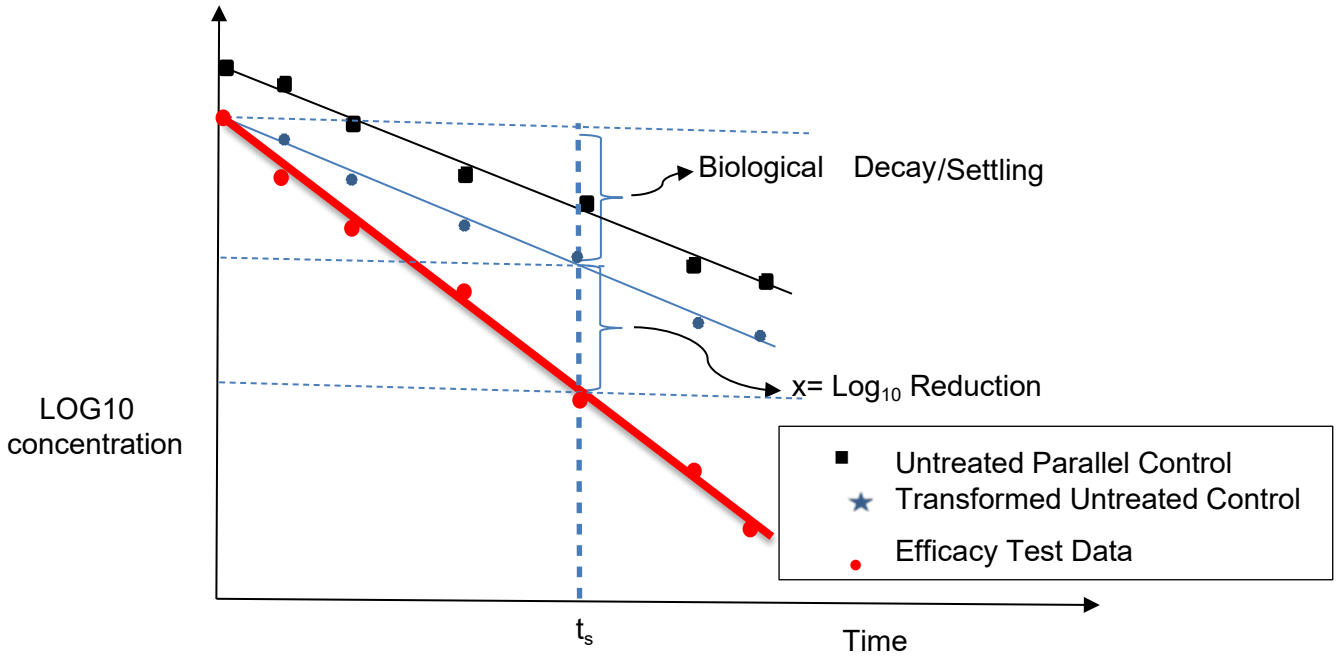

Figure 2: Schematic of log reduction and biological decay

Log_{10} reduction is used to evaluate the substance efficacy, if the $\text{Log Reduction} \geq 3.0$ in a ≥5 minute contact time, the technology is considered to meet the current performance criterion.

Appendix 4: Glossary & Abbreviations

GLOSSARY

	Definition
Aerobiology	Study of the behavior of microorganisms, pollen and allergens in air
Air sanitization	Removing and/or inactivating potentially harmful microorganisms in air
Nebulizer	Any device capable to turning a powder or liquid into airborne particles
Nebulizer fluid	A suspension of the test microorganism in a soil load and an antifoam
Refrigerated stock	A prepared microbial suspension used to initiate cultures for use in experimentation
Slit-to-agar (STA) air sampler	A device where airborne microbes are drawn through a narrow slit for capture on a nutrient recovery medium by impaction
Soil load	A mixture of one or more organic/inorganic substances added to suspensions of test microbes to simulate the presence of bodily secretions, excretions, or other materials that may shield microbes by interfering with the activity of a microbicidal agent.

Abbreviations

µL	Microliter
BSA	Bovine serum albumin
BSC	Biological safety cabinet
BSL	Biosafety level
DDW	Deionized distilled water
m^3	Cubic meter
h	Hour
HEPA	High-efficiency particle arrestor
ID	Inside diameter
kPa	Kilo-Pascal
m^3	Cubic meter
PBS	Phosphate-buffered saline
PES	Polyethersulfone
PPE	Personal protective equipment
PVC	Polyvinyl chloride
QAU	Quality assurance unit
SD	Standard deviation
STA	Slit-to-agar
TSA	Trypticase soy agar
TSB	Trypticase soy broth
v/v	Volume/volume

Appendix 5

Example of Aerobiological Testing Schedule

Example of daily activities to assess chemical-based formulations for indoor air sanitization

Description	Week 1					Week 2					Week 3					Week 4				
	Day					Day					Day					Day				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Staphylococcus aureus:																				
Stability in Air	√	√																		
Neutralization	√																			
Baseline + Lot 1		√	√√																	
Baseline + Lot 2				√√	√															
Baseline + Lot 3					√	√√														
Klebsiella pneumoniae																				
Stability in Air							√	√												
Neutralization							√													
Baseline + Lot 1								√	√√											
Baseline + Lot 2										√√	√									
Baseline + Lot 3											√	√√								
Pseudomonas aeruginosa																				
Stability in Air													√	√					√	
Neutralization													√							
Baseline + Lot 1														√	√√					
Baseline + Lot 2																√√	√			
Baseline + Lot 3																	√	√√		

Key: Sa (*Staphylococcus aureus*); Kp (*Klebsiella pneumoniae*); Pa (*Pseudomonas aeruginosa*); C (Stability-in-air); NE (neutralization effectiveness control); Rep (replicate); B (Batch); Air-sampling by STA (slit-to-agar) sampler.

Daily activities for a given lot/microorganism/replicate

Day 1 – AM

- 9:00 –Wear PPE, check the chamber for integrity as well as air temperature and RH, adjust RH if required. Seal the door of the chamber. Prepare suspension for nebulization. Collect the nebulizer fluid for CFU assay.
- 10:00- Nebulize test bacteria for control counts (Stability-in-Air) [10-min nebulization + 5-min stabilization + collecting at least four air samples over the next 60-min+ collect the nebulizer fluid for CFU assay.]
- 11:15 – Stability-in-Air (untreated control) assessment complete

Keep the prepared bacterial culture on ice for use later during the day

- 11:15 – 12:00 – Evacuate the chamber. Dilution and plating of nebulizer fluid samples. Incubation of the culture plates. Observe plates from previous days' experiments and take photographs, clean up and decontaminate lab ware.
- 12:00 – 1:00 PM – Continue Evacuating the chamber
- 12:00 – 1:00 – Scientists take break plus flush the chamber

Day 1 – PM

- 1:00 PM–Wear PPE, check the chamber for integrity as well as air temperature and RH, adjust RH if required. Seal the door of the chamber. Prepare suspension for nebulization. Collect the nebulizer fluid for CFU assay.
- 2:00 PM - Nebulize test bacteria
- 2:15 PM – Dispense product
- 2:25 – 3:00 PM – Collect air-sample by STA, remove plate for incubation ➢ 3:00 PM test completed
- 3:00 – 5:00 PM – Evacuate the chamber. Dilution and plating of nebulizer fluid samples. Incubation of the culture plates. Decontaminate the labware/waste, prepare culture/supplies for next day and exit BSL-2 facility